

# STAIN COMBINATIONS IN LIVING INSECTS

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The commonly practiced method in stain technique on non-living material is the use of two types of stains. This proves valuable for differentiation of portions of the tissue, for example, setting off the nucleus of the cell from the remainder of the protoplasmic material, as well as other portions of cellular structure. From past experience standards have been set up whereby certain stains are suggested for certain cellular structures or tissues. In the test series discussed in this paper the two general factors taken into account (besides the choosing of stains previously found good for viable tissue) were the acidity or basidity of the stain materials and the coloration, either of a red type or of a blue type.

The present paper is an expansion of the method developed by the author (1946<sup>1</sup>) for differentiating organs and tissues by injection of dilute stains in living animals. In the present experimental series two stains instead of one were used. The series was selected so that in each combination of two stains, one would be of a shade of red and one blue and at the same time one would be acidic in composition and one would be basic. Curiosity was the factor which prompted the author to use red-blue, acidic-basic mixtures of stains to see what shades of color might be obtained upon gross examination of the body organs and tissues and what dominance of one shade over another would appear in such a mixing process. Each individual staining solution was not tested to determine its relative acidity or basidity, but was used from its general classification as an acid or base material. Normal saline (0.85% aqueous solution of sodium chloride) was used as the medium for all the materials.

The materials used were those previously found useful as vital stains with definite color properties and not seemingly too injurious to the insect tissues. Twelve stain materials (dry) were chosen, made into solutions individually with normal saline as the medium, and mixed immediately before injection into the insect. Some difficulty was encountered in the beginning of the work due to the chemical activity of some materials upon mixture; some, allowed to stand for as much as five or ten minutes, would form a thickened or congealed solution which proved difficult to inject as well as toxic to the experimental animals. For this reason the practice of mixing the stain solutions just before injection by the hypodermic needle was used, and, except in four cases, no further serious difficulty was encountered.

The stains used are listed below, followed by their acidic or basic classification:

Bordeaux Red (acidic).	Blue de Lyon O (basic).
Rose Bengal (acidic).	Brilliant Cresyl Blue (basic).
Biebrich Scarlet (acidic).	Azur II (basic).
Congo Red (acidic).	Toluidin Blue (basic).
Safranine O (basic).	Aniline Blue (acidic).
Fuchsin Basic (basic).	Trypan Blue (acidic).

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<sup>1</sup>Research work done with the guidance of Dr. C. H. Kennedy, of the Department of Entomology, Ohio State University.

These were used to make twenty combinations (by twos):

Bordeaux Red x Blue de Lyon O.	Rose Bengal x Azur II.
Rose Bengal x Brilliant Cresyl Blue.	Rose Bengal x Toluidin Blue.
Biebrich Scarlet x Azur II.	Biebrich Scarlet x Blue de Lyon O.
Congo Red x Toluidin Blue.	Biebrich Scarlet x Brilliant Cresyl Blue.
Aniline Blue x Safranin O.	Biebrich Scarlet x Toluidin Blue.
Trypan Blue x Fuchsin Basic.	Congo Red x Blue de Lyon O.
Bordeaux Red x Brilliant Cresyl Blue.	Congo Red x Brilliant Cresyl Blue.
Bordeaux Red x Azur II.	Congo Red x Azur II.
Bordeaux Red x Toluidin Blue.	Aniline Blue x Fuchsin Basic.
Rose Bengal x Blue de Lyon O.	Trypan Blue x Safranin O.

Each stain material was weighed and combined with normal salt solution at the rate of 1 gram dry material to 100 cc. normal saline. A small amount of each staining solution used in a combination was mixed with a similar amount of the other material used and the injections of .1 cc. were taken from this mixture. An experimental attempt to inject each stain solution separately proved destructive to the experimental insects both from the puncturing of the body wall by the hypodermic needle and the excessive amount of material introduced into the body cavity.

The experimental insect used in all the work was the American cockroach (*Periplaneta americana*). Under six months of age, the roaches do not prove to be good experimental subjects, consequently only those over six months or fully mature adults were used. These were taken from laboratory cultures where the age could be determined to a close degree.

After the solutions were made from the dry materials seven of them (Brilliant Cresyl Blue, Blue de Lyon O, Trypan Blue, Toluidin Blue, Azur II, Aniline Blue and Congo Red) made homogeneous mixtures as discerned by the eye. The last, Congo Red, was rather thick, and upon revolving the container rather large clumps of material were found clinging to the glass walls. Some settling occurred in Safranin O, Biebrich Scarlet and Bordeaux Red, the last only to a slight degree. In the case of Fuchsin Basic and Rose Bengal the solution appeared thin upon standing for a period of time with a large amount of settling in the bottom of the bottle. While all were shaken before use, these last two mentioned required additional agitation to thoroughly mix the solution. Upon examination all these stain solutions appear to be suspensions of greater or lesser degree rather than true dissolved materials. This is especially noted in the cases where settling occurred.

In the combinations made from the stain solutions those containing Congo Red and Toluidin Blue proved to be the most difficult in the attempts to obtain mixtures. Five of them were definitely thick, viscous liquids. These were Congo Red and Toluidin Blue, Bordeaux Red and Toluidin Blue, Biebrich Scarlet and Toluidin Blue, Congo Red and Blue de Lyon O, Congo Red and Brilliant Cresyl Blue. The remainder of the combinations formed more or less free liquid mixtures, although the appearance of granules or small flakes of material appeared in many of them. The ease of mixing and the thickness of the resulting combination may well be a very important factor in the value of the stain combination from the standpoint of mortality as well as coloring of structures.

#### METHODS

Insects of proper age were chosen from the laboratory cultures, indiscriminately as to sex, and removed to an anesthetizing jar. A piece of cotton or cheesecloth saturated with ethyl ether was placed in the jar, a tight cover placed over the top, and the insects left until rapid movement of the legs had ceased. By this time the insects were usually lying on their backs and producing slight movements with their bodies and legs. This was done only when everything was ready for the

injections to be made because the roaches will recover quickly when they are the recipients of fresh air.

When calmed so that rapid movements of the legs, etc., would not impede the insertion of the hypodermic needle, the insects were taken out of the anesthetizing chamber and .1 cc. of the mixture to be tested was placed in the body cavity by puncturing the thin cuticle at the base of the third abdominal segment, using a 1 cc. hypodermic with a very fine needle. A rather slow release of the solution in the barrel proves best to prevent injury to internal organs.

The insects were then placed in "housing jars" (i. e., small culture jars) to await the time of dissection which was set at one-half hour, one day, and two days from the time of injection. Before dissection ether was again used to stop rapid movement until the legs could be removed and the ventral wall opened and spread apart on a small dissecting tray. Examination took place under a binocular microscope, as well as with the naked eye. The results of the gross examination of the internal structures is recorded for each stain combination.

### RESULTS

The stains used had been selected with the view in mind of their good record previously of low mortality. However, some trouble was encountered in the use of four combinations. These were Safranin O and Aniline Blue, Rose Bengal and Toluidin Blue, Congo Red and Blue de Lyon O, Congo Red and Azur II. While no difficulty was apparent at one-half hour intervals, attempts to have survival of injected insects for one and two day periods necessitated many more trials than was the case with any of the others of the series.

Certain expressions used in connection with the descriptions of stain results to identify structures are of common recognition and do not need further explanation while others are those used by the author to clarify differentiated areas. The terms salivary glands, oesophagus, stomach, gizzard, malpighian tubules, tracheae, fat bodies, leg muscles, femurs, ovaries, testes, gastric caecae, ventral nerve cord are ones easily identified in the roach from a previous study of its internal structures or those of many of the other common insects. The terms "dorsal wall material" and "ventral wall material" refer to the fatty tissue lying next to the body wall of the respective surfaces. These quite often appear to be the same color as the fat bodies lying in the abdomen, but in a few cases differ in shade or color. The intestine portion of the digestive tract has been divided into three determinations. The "entire intestine" is used when the same shade is found throughout the entire length of the intestine; "upper intestine" for the upper half (using caudal-anal direction) and "lower intestine" for the lower half (if a smaller area is considered, it is designated as one-third, one-fourth, etc.). The words "heart outline" are used when a color appears to form an outlying band along either side of the heart area. The term "entire heart" includes both the true heart and the dorsal aorta, "true heart" the heart proper, and "dorsal aorta" the portion usually designated as such. "Heart wing muscles" is used for those V-shaped muscles, whose base is toward the heart, extending from the heart outward along the tracheae.

Each of the stain combinations were tested over a period of two days at the most. Dissections and examinations were made at intervals of one-half hour, one day and two days. In the records of this testing, the descriptions have been arranged in such a way that the time interval giving the best results from the standpoint of color variety and the number of structures shown to be affected is given first under the appropriate heading, with the introductory words *Best timing*. In this portion each of the organs and tissues affected are presented with the color noted in the examination. This part is followed by information of the other two time intervals with the structures affected and the colors represented merely listed.

In a review of the various combinations it has been noted that only three out of the twenty used showed a dominance of blue shades, i. e., including blue, violet, etc., more than red, rose, pink, etc. Toluidin Blue was the "blue" stain used in each of these (Congo Red x Toluidin Blue, Bordeaux Red x Toluidin Blue, Biebrich Scarlet x Toluidin Blue). In nine combinations the "red" predominated and in eight the red and blue shades were about equal. It might be noted that two, Safranin O and Aniline Blue, were used only twice each in combination with another stain, and the red shades were dominant in the resulting four sets of the test series. In all the combinations lavender was one of the colors appearing on the affected structures. No other color or shade was found throughout the entire series.

#### ROSE BENGAL X TOLUIDIN BLUE

*Best timing:* One day—salivary glands, deep lavender; oesophagus, stomach, gastric caecae, light violet; gizzard, violet; intestine, violet to lavender; malpighian tubules, deep red (wine) or deep purple; heart outline, heavy purple, with outer edge lavender; entire heart, ventral nerve cord, rose; dorsal wall material, rosy-violet or fuchsia; ventral wall material, fuchsia; fat bodies, bright blue with some lavender shading; leg muscles, femurs, lavender; ovaries, rose to lavender.

Half hour—Structures: salivary glands, oesophagus, stomach, gizzard, gastric caecae, intestine, malpighian tubules, entire heart, dorsal and ventral wall material, fat bodies, leg muscles. Colors: violet, lavender, blue.

Two days—Structures: salivary glands, stomach, gizzard, gastric caecae, intestine, malpighian tubules, entire heart, dorsal wall material, fat bodies, leg muscles. Colors: lavender, pink, purple, violet, rose, blue.

#### ROSE BENGAL X BLUE DE LYON

*Best timing:* One day—salivary glands, lavender; stomach, gizzard, violet tinge; gastric caecae, light violet; intestine, mostly violet; malpighian tubules, one-half scarlet; entire heart, pink and violet blended (whole looks purple); dorsal wall material, deep violet; fat bodies, one-third violet, rest bright pink; spermatheca, dark lavender to violet.

Half hour—Structures: salivary glands, oesophagus, stomach, gizzard, gastric caecae, intestine, entire heart, dorsal wall material, fat bodies, leg muscles, testes. Colors: lavender, violet, dark red, scarlet, purple, orange.

Two days—Structures: salivary glands, stomach, gastric caecae, intestine, malpighian tubules, entire heart, dorsal wall material, fat bodies, ventral wall material. Colors: lavender, rose, scarlet.

#### BORDEAUX RED X TOLUIDIN BLUE

*Best timing:* Two days—Gizzard, intestine, deep purple; gastric caecae, dark lavender (extended); malpighian tubules, deep wine red; entire heart, deep purple (color flakes present); dorsal wall material, greenish blue; fat bodies, mostly pale blue; leg muscles, femurs, dull green; ovaries, yellow-green; ovarian tubes, light to median blue; ventral nerve cord, blue tinged, darker at ganglia.

Half hour—Structures: salivary glands, gastric caecae, malpighian tubules, entire heart, dorsal wall material, fat bodies. Colors: purple, blue, lavender, rosy-lavender, violet, pink.

One day—Structures: salivary glands, stomach, gastric caecae, upper intestine, malpighian tubules, heart outline, dorsal wall material, fat bodies. Colors: purple, blue, lavender, red, violet.

#### SAFRANINE O X TRYPAN BLUE

*Best timing:* Two days—salivary glands, light lavender; gizzard, upper part pink, lower part lavender; gastric caecae, leg muscles, deep rose; intestine, rosy

lavender tinge; malpighian tubules, one-half rose; heart outline, heart wing muscles, violet; entire heart, dark purple; dorsal wall material, salmon (some pink); ventral wall material, lavender; fat bodies, pink; ovaries (and tubes), rosy lavender.

Half hour—Structures: salivary glands, stomach, gizzard, intestine, malpighian tubules, entire heart, dorsal wall material, fat bodies, leg muscles, testes. Colors: lavender, rosy lavender, red, pink, salmon.

One day—Structures: salivary glands, stomach, gastric caecae, upper intestine, entire heart, dorsal wall material, fat bodies. Colors: pink, lavender.

#### BIEBRICH SCARLET X BRILLIANT CRESYL BLUE

*Best timing:* Half hour—salivary glands, light violet; stomach, reddish; gizzard, lavender; gastric caecae, slightly reddish; entire heart, heart wing muscles, dark purple; dorsal wall material, bright pink to lavender; fat bodies, pink, lavender and violet; leg muscles, bright pink streaks; testes, pale to bright blue.

One day—Structures: salivary glands, malpighian tubules, entire heart and outline, dorsal wall material, fat bodies, leg muscles, ovaries. Colors: pink, dark red, brown, orange, blue, lavender.

Two days—Structures: salivary glands, gizzard, gastric caecae, upper intestine, malpighian tubules, entire heart, dorsal wall material, fat bodies. Colors: lavender, pink, red, brown, blue, salmon.

#### CONGO RED X TOLUIDIN BLUE

*Best timing:* Two days—salivary glands, faint blue tinge; stomach, lower one-third light lavender; gizzard, lavender; gastric caecae, faint lavender tinge at base; intestine, deep lavender with some light purple portions; malpighian tubules, lavender (granular appearance); heart outline, heavy dark purple with heavy violet accumulation along edge; heart wing muscles, purple to violet; dorsal wall material, very bright blue, some bright violet immediately along tracheae; ventral wall material, yellowish blue; fat bodies, mostly pale to bright blue in abdomen, few light violet spots. Presence of dark purple flakes noted in center of body.

Half hour—Structures: gastric caecae, intestine, fat bodies, testes. Colors: lavender, purple, blue, red.

One day—Structures: salivary glands, stomach, gizzard, gastric caecae, intestine, heart outline, dorsal and ventral wall material, fat bodies. Colors: blue, lavender, violet, black.

#### BIEBRICH SCARLET X BLUE DE LYON

*Best timing:* One day—salivary glands, stomach, lavender; gizzard, gastric caecae, leg muscles, light lavender; upper intestine, deep lavender (almost purple); malpighian tubules, orange-red; entire heart, deep blue; dorsal wall material, lavender tinges; ventral wall material, mostly deep blue.

Half-hour—Structures: salivary glands, stomach, gastric caecae, dorsal aorta, dorsal wall material. Colors: lavender, orange, pink.

Two days—Structures: stomach, intestine, malpighian tubules, entire heart, dorsal wall material, fat bodies. Colors: blue, lavender, orange-red, pink.

#### FUCHSIN BASIC X TRYPAN BLUE

*Best timing:* Two days—salivary glands, lavender; gizzard, bluish tinge; gastric caecae, lavender tinge; malpighian tubules, lavender to scarlet (granular); dorsal aorta, femurs, purple; heart wing muscles, purple; fat bodies, faint blue tinge in thorax, some lavender tinge in abdomen; leg muscles, deep blue.

Half-hour—Structures: salivary glands, stomach, malpighian tubules, dorsal aorta, testes. Colors: lavender, rosy lavender, pink, scarlet.

One day—Structures: salivary glands, gizzard, gastric caecae, malpighian tubules, dorsal aorta, dorsal wall material, leg muscles, ovaries. Colors: lavender, blue, scarlet.

**BORDEAUX RED X BRILLIANT CRESYL BLUE**

*Best timing:* Half hour—salivary glands, deep bright blue; gizzard, lavender tinge; gastric caecae, purple tinge (enlarged); malpighian tubules, dull wine red; entire heart, red tinge; heart wing muscles, deep purple with spot of orange-red in center; fat bodies, in abdomen ventral side pink to deep lavender, dorsal side pink, bright pink and lavender; leg muscles, deep blue; oviducts, light blue.

One day—Structures: salivary glands, gastric caecae, upper intestine, malpighian tubules, heart outline, dorsal wall material, fat bodies. Colors: pink, scarlet, red, violet, purple, lavender, blue.

Two days—Structures: malpighian tubules, heart outline. Colors: red, lavender.

**ROSE BENGAL X AZUR II**

*Best timing:* Half hour—salivary glands, lavender; stomach, partially violet; gastric caecae, dark orange; intestine, orange; malpighian tubules, rose and orange; entire heart, purple; heart wing muscles, light purple; dorsal wall material, rose-pink to lavender; fat bodies, bright rose (almost scarlet) to violet.

One day—Structures: salivary glands, gizzard, gastric caecae, upper intestine, malpighian tubules, entire heart, dorsal wall material, fat bodies, ovaries. Colors: lavender, pink, rose.

Two days—Structures: gizzard, gastric caecae, intestine, malpighian tubules. Colors: blue, rose, violet.

**CONGO RED X BLUE DE LYON**

*Best timing:* Half hour—salivary glands, lavender; stomach, gizzard, intestine, gastric caecae, dark red-brown; entire heart, heart wing muscles, maroon; dorsal wall material, dark purple; fat bodies, pink or lavender tinged; testes, dull pink.

One day—Structures: salivary glands, stomach, malpighian tubules, entire heart, dorsal wall material, fat bodies, gastric caecae. Colors: lavender, rust (orange-brown), salmon, orange. General appearance is salmon.

Two days—Structures: salivary glands, stomach, gizzard, intestine, malpighian tubules, heart outline, fat bodies, gastric caecae. Colors: lavender, blue.

**SAFRANINE O X ANILINE BLUE**

*Best timing:* One day—salivary glands, lavender (with bright orange "veins"); gastric caecae, bright rose; intestine, one-half rosy lavender; malpighian tubules, bright orange-red; entire heart, lavender tinge; heart wing muscles, light lavender; dorsal wall material, salmon to median orange; fat bodies, salmon; leg muscles, deep pink; ovarian tubules, partially blue; nerve cord ganglia, pink tinge. Overall appearance is salmon.

Half hour—Structures: salivary glands, stomach, gastric caecae, malpighian tubules, dorsal wall material, fat bodies. Colors: pink, orange.

Two days—Structures: stomach, gastric caecae, intestine, dorsal and ventral wall material. Colors: bluish-green, orange-red, pink, lavender, rose.

**FUCHSIN BASIC X ANILINE BLUE**

*Best timing:* Two days—salivary glands, very light blue; stomach, spots of blue and lavender; gizzard, gastric caecae, medium blue; intestine, dark lavender to purple or blue; malpighian tubules, light purple or fuchsia (with minute dark granules); entire heart, dorsal wall material (mostly), testes, light blue; heart wing muscles, very light blue.

Half hour—Structures: salivary glands, stomach, gizzard, gastric caecae, malpighian tubules, entire heart, dorsal wall material. Colors: blue, lavender, orange-brown, pink, rose.

One day—Structures: salivary glands, gizzard, lower intestine, malpighian tubules, entire heart, dorsal wall material, femurs. Colors: blue, lavender, scarlet.

**BIEBRICH SCARLET X TOLUIDIN BLUE**

*Best timing:* One day—salivary glands, very dark lavender; stomach, lower one-half light purple; gizzard, lavender; gastric caecae, tips violet; intestine, small portion blue with rest lavender; malpighian tubules, uncolored or with some portions deep wine red; heart outline, violet; heart wing muscles, heavy violet; dorsal wall material, mostly light blue; fat bodies, creamy, pale, medium or dark blue or violet. Globules of dark blue material through body.

Half hour—Structures: salivary glands, stomach, gizzard, intestine, entire heart, dorsal wall material, fat bodies, leg muscles. Colors: violet, purple, blue.

Two days—Structures: gizzard, gastric caecae, intestine, malpighian tubules, heart outline, dorsal wall material, fat bodies. Colors: lavender, purple, blue, violet.

**ROSE BENGAL X BRILLIANT CRESYL BLUE**

*Best timing:* Half hour—salivary glands, deep rose-lavender; gastric caecae, slight pink tinge; malpighian tubules, pale to light, bright blue; dorsal aorta, anterior one-fourth rose, remainder deep lavender; dorsal wall material, rose lavender; fat bodies, posterior one-third (abdomen) pink, rest deep rose and lavender combination; leg muscles, light blue tending to lavender; femurs, scarlet.

One day—Structures: gizzard, gastric caecae, intestine, malpighian tubules, entire heart, fat bodies. Colors: lavender, red, light blue, rose.

Two days—Structures: gastric caecae, upper intestine, heart outline, dorsal wall material, fat bodies. Colors: red, pink, brown, blue.

**BORDEAUX RED X BLUE DE LYON**

*Best timing:* One day—salivary glands, lavender (with blue "veins"); stomach, fat bodies, dark blue streaked; intestine, traces of light violet; malpighian tubules, light rosy lavender; heart outline, heavy blue coloring at entrances of tracheae (granules present); dorsal wall material, some pale lavender tinges with some scattered blue spots; ovaries, deep lavender with few blue spots.

Half hour—Structures: salivary glands, lower intestine, malpighian tubules, heart outline and entire heart, fat bodies. Colors: lavender (pale and reddish), blue, red, scarlet.

Two days—Structures: gastric caecae, malpighian tubules, heart outline, fat bodies, ovaries. Colors: blue, scarlet, dull red, lavender.

**BORDEAUX RED X AZUR II**

*Best timing:* One day—gastric caecae, light green tinge; intestine, mostly purple; malpighian tubules, one-half very faint pink; heart outline, brown pigmented; dorsal aorta, orange-pink; heart wing muscles, light tan; fat bodies, tinges of light blue and light pink.

Half hour—Structures: salivary glands, stomach, malpighian tubules, entire heart, dorsal wall material, testes. Colors: lavender, pink, red, blue.

Two days—Structures: gastric caecae, malpighian tubules, intestine. Colors: red, bright yellow, violet, lavender.

**CONGO RED X AZUR II**

*Best timing:* Two days—gastric caecae, upper intestine, faint pink; heart outline, dark brown (flaky material); heart wing muscles, light orange-brown; dorsal wall material, salmon (scattered spots between tracheae); ovaries, few orange spots. Clumps and flakes of very dark reddish brown material through body cavity.

Half hour—Structures: salivary glands, entire heart. Colors: pink, yellowish brown. Brown flakes scattered through body.

One day—Structures: salivary glands, upper intestine, entire heart, dorsal and ventral wall material, fat bodies, leg muscles. Colors: blue, lavender, violet, rosy

lavender. Dark violet (crystalline) material found in spots on dorsal side of fat material.

**CONGO RED X BRILLIANT CRESYL BLUE**

*Best timing:* One day—salivary glands, pale lavender; heart outline, heavy brown pigment; dorsal aorta, some orange (deeper than normal); heart wing muscles, orange-brown (brown specks present); dorsal wall material, salmon; oesophagus, stomach, gizzard, gastric caecae, intestine, orange. Impression of body cavity taken as a whole is of a salmon color; this is especially true to the naked eye.

Half hour—Structures: salivary glands, entire heart, dorsal wall material, fat bodies. Colors: lavender, violet, red, pink. Dark brown flecks through body cavity.

Two days—Structures: salivary glands, gastric caecae, intestine, malpighian tubules, entire heart, dorsal wall material, fat bodies. Colors: pink, orange-red, red, orange, orange-yellow. Brown pigment through body cavity. Body cavity as a whole looks orange.

**BIEBRICH SCARLET X AZUR II**

*Best timing:* Half hour—salivary glands, light, bright blue; malpighian tubules, few posterior ones light blue, few dull red (with minute red granules); heart wing muscles, purple; fat bodies, portions of pink and blue (light); leg muscles, pale blue; testes, light blue.

One day—Structures: stomach, intestine, malpighian tubules, testes. Colors: lavender, deep red, rose.

Two days—Structures: gastric caecae, intestine, malpighian tubules, heart outline. Colors: purple, violet.

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